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T-cells subsets and activation in bronchial mucosa of sensitized Brown–Norway rats after single allergen exposure

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SUMMARY

We have investigated the relationship between changes in T-cell activation in the bronchial mucosa, airway responsiveness and eosinophilic inflammation in sensitized Brown–Norway rats exposed to ovalbumin (OVA). Rats sensitized intraperitoneally with OVA and exposed to OVA aerosol 21 days later showed an enhanced increase in lung resistance (RNL) to acetylcholine ($P < 0.05$), and a significant increase in the number of eosinophils, neutrophils and lymphocytes in bronchoalveolar lavage fluid (BAL) ($P < 0.05$), compared with sensitized but saline-exposed controls. There was a significant increase in cells expressing the T-cell activation marker CD25 ($P < 0.05$) and the numbers of $CD8^+$ T cells ($P < 0.05$), but not in the numbers of $CD2^+$ and $CD4^+$ cells. Eosinophil counts in airway submucosal tissue, as assessed by staining with BMK-13; a monoclonal antibody that binds to eosinophil major basic protein (MBP), were increased in rats receiving sensitization and exposure to OVA compared with naive controls ($P < 0.002$). There were significant positive correlations between the increase in R_L to acetylcholine and the numbers of $CD25^+$ ($r = 0.92$, $P < 0.001$), $CD4^+$ ($r = 0.77$, $P < 0.05$), $CD8^+$ ($r = 0.71$, $P < 0.05$) and MBP^+ ($r = 0.72$, $P < 0.03$) cells in the OVA-sensitized and exposed group, but not in saline-exposed or naive animals. The number of MBP^+ cells also correlated with CD25 expression ($r = 0.71$, $P < 0.05$). We conclude that airway hyper-responsiveness and inflammatory cell infiltration caused by OVA exposure of sensitized animals is associated with the presence of activated T cells in the airway mucosa. $CD8^+$ T cells may play a role in the regulation of events leading to eosinophil inflammation and airway hyper-responsiveness.

INTRODUCTION

The mechanisms regulating inflammatory changes that have been associated with airway hyper-responsiveness in asthma remain poorly understood. More recently, it has become clear that T cells can have direct proinflammatory roles and may express and generate cytokines that are capable of attracting and activating potential effector cells such as eosinophils and monocytes.[1] Studies of bronchial mucosal biopsies obtained from allergic asthmatic patients have demonstrated an increased number of T lymphocytes, with evidence of activation;[2,3] in addition, an increased number of activated T cells bearing the CD25 marker has been observed in cells obtained by bronchoalveolar lavage.[4] The number of activated T cells correlated with asthma severity and with the number of activated tissue eosinophils.[3] The T cells were shown to express mRNA for interleukin-5 (IL-5), a potent chemoattractant and activator of eosinophils.[4,5] These observations suggest that activated T cells may induced eosinophilia, and may underlie asthma severity.

In order to investigate further the potential role for activated T cells to modulate airway hyper-responsiveness, we have examined the relationship between T-cell subsets ($CD4^+$ and $CD8^+$), activated T cells ($CD25^+$), eosinophils and airway responses to inhaled acetylcholine in sensitized Brown–Norway rats exposed to a single challenge of ovalbumin (OVA). In previous studies, we have demonstrated that Brown–Norway rats develop a transient episode of airway hyper-responsiveness within 24 hr of OVA challenge, associated with an increased number of eosinophils and activated T lymphocytes bearing the CD25 marker recovered by bronchoalveolar lavage (BAL).[6,7] Moreover, the cell influx and airway hyper-responsiveness were abolished by corticosteroids.[8] The present study was conducted to characterize further our Brown–Norway rat model.

MATERIALS AND METHODS

Animals, sensitization procedures and allergen exposure

Virus-free inbred male Brown–Norway rats (Harlan Olac Ltd, Bicester, UK) (180–230 g) were kept in a special caging system (Maximiser; Theseus Caging Systems Inc., Hazleton, PA). Rats were injected with OVA (1 ml, 1 mg/ml) in $Al(OH)_3$

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(100 mg/ml) in 0·9% saline intraperitoneally (i.p.) on 3 consecutive days. Aerosol exposure was accomplished by placing the rats in a 6·5-l plexiglass chamber connected to a DeVilbiss PulmoSonic nebulizer (model No. 2512; DeVilbiss Health Care Ltd, Feltham, UK) that generated an aerosol mist pumped into the exposure chamber by the airflow supplied by a small animal ventilator set at 60 strokes/min with a pumping volume of 10 ml. Rats were exposed to 1% (w/v) OVA for 15 min.

Three groups of rats were studied.

Naive rats. Non-sensitized and non-exposed animals were kept in identical conditions to the sensitized animals for 3 weeks and studied thereafter at the same time points ($n = 8$).

Sensitized and saline-exposed rats. One millilitre of 1 mg OVA/100 mg $Al(OH)_3$ in a 0·9% (w/v) saline suspension was injected i.p. for 3 consecutive days. Three weeks after the final injection rats were exposed to saline aerosol for 15 min ($n = 8$). Animals were then studied 18–24 hr after exposure.

Sensitized and OVA-exposed rats. One millilitre of 1 mg OVA/100 mg $Al(OH)_3$ in a 0·9% (w/v) saline suspension was injected intraperitoneally for 3 consecutive days. Three weeks after the final injection animals were exposed to 1% OVA aerosol for 15 min ($n = 8$). Animals were studied 18–24 hr after exposure.

Measurement of bronchial responsiveness

Twenty-four hours after OVA aerosol exposure, anaesthetized (60–80 mg/kg pentobarbitone, i.p.), rats were tracheostomized and ventilated (10 ml/kg; 90 strokes/min) using a small animal respirator (Harvard Apparatus Ltd, Edenbridge, UK). Transpulmonary pressure was measured with a pressure transducer (model FCO 40; ± 1000 mm of H_2O; Furness Controls Ltd, Bexhill, UK) with one side attached to an air-filled catheter inserted into the right pleural cavity, and the other side attached to a catheter connected to a side-port of the intratracheal cannula. Airflow was measured with a penumotachograph (model F1L; Mercury Electronics Ltd, Glasgow, UK) connected to a transducer (model FCO 40; ± 20 mm of H_2O; Furness Controls Ltd). The signals from the transducers were digitalized with a 12-bit analogue-digital board (NB-MIO-16; National Instruments, Austin, TX) connected to a Macintosh II computer (Apple Computer Inc., Cupertino, CA) and analysed with software (LabView; National Instruments, Austin, TX) that was programmed to calculate lung resistance (R_L) instantaneously.

Aerosols (mean mass diameter: 3·8 μm; geometric SD: 1·3) were generated with an ultrasonic nebulizer (model no. 2512; DeVilbiss Health Care). Following propanolol (1 mg/kg intravenously) to inhibit adrenergic effects, 0·9% NaCl (45 breaths) was administered, and the subsequent lung resistance value was used as baseline. Increasing half-$\log_{10}$ concentrations of acetylcholine (ACh; 45 breaths) were administered at 5–7-min intervals, with one hyperinflation of twice the tidal volume applied between each ACh concentration. PC_{50} and PC_{100} (concentration of ACh needed to increase lung resistance by 50% and 100% above baseline, respectively) were calculated by log–linear interpolation of concentration–response curves from individual animals.

Bronchoalveolar lavage and cell counting

This has been described in detail elsewhere.[6] Briefly, lungs were lavaged with 20 ml of a total volume of sterile saline at room temperature. Total cell counts were determined by Kimura staining of the samples[9] and counting in a Neubauer chamber under a light microscope. Differential cell counts were made from cytospin preparations stained by May–Grünwald stain. Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard morphology, and 500 cells were counted for this purpose under × 400 magnification. The percentage and absolute numbers for each cell type were calculated.

Collection of lung tissues

The thoracic cavity was opened and the lungs were removed and inflated with 5 ml saline/OCT (1 : 1). Five-mm^3 blocks of the left lung tissue around the main bronchus were cut, embedded in OCT medium (Raymond A. Lamb, London, UK) and snap-frozen to −80° using isopentane (BDH, Poole, UK) and liquid nitrogen. Cryostat sections (6 μm) of the tissues were cut, air-dried and fixed in absolute alcohol for immunocytochemistry, and in formol–saline (10%) for carbol chromotrope staining. Slides were then air-dried, wrapped in aluminium foil and stored at −80° until immunohistochemistry was performed.

Immunohistochemistry

Cryostat sections of bronchial and lung tissue were incubated with mouse monoclonal antibodies (mAb) raised against rat antigens and conjugated to biotin (Cambridge Biosciences, Cambridge, UK). Anti-rat CD2 (OX34) at a dilution of 1 : 30, anti-rat CD4 (OX35) at a dilution of 1 : 30, anti-rat CD8 (OX8) at a dilution of 1 : 40, anti-rat CD25 (OX39) at a dilution of 1 : 5 and anti-rat CD45RC at a dilution of 1 : 50 were applied for 1 hr at room temperature. After washing in Tris buffer for 15 min, the second layer was applied, which was streptavidin–alkaline phosphatase (1 : 200; Amersham Int., Amersham, UK) for 15 min at room temperature. Specifically bound alkaline phosphatase was detected as a red colour following a 20-min incubation with Naphthol AS-MX phosphate in 0·1 M trismethylamine–HCl buffer (pH 8·2), containing 0·01 M levamisole to inhibit endogenous alkaline phosphatase and 1 mg/ml Fast Red-TR salt.

The use of the in-house mAb BMK-13 was adapted for the detection of human eosinophil major basic protein (MBP). This mAb gives more sensitive and specific staining of eosinophils in frozen sections compared with carbol chromotrope 2R or haematoxylin and eosin staining. Cryostat sections of bronchial and lung tissue were incubated with the mAb BMK-13. This is a mouse IgG1 hybridoma clone raised against human MBP.[10] Recent studies have validated BMK-13 binding to rat eosinophils in cryostat sections of rat airways (Q. Hamid & R. Moqbel, unpublished data). BMK-13 was used at a dilution of 1 : 30 for 30 min at room temperature. After labelling with the second layer (rabbit anti-mouse IgG), positive staining was visualized by the alkaline phosphatase–anti-alkaline phosphatase technique. Specifically bound alkaline phosphatase was detected as a red colour following incubation with Naphthol AS-MX phosphate in 0·1 M trismethylamine–HCl buffer (pH 8·2), containing levamisole to inhibit endogenous alkaline phosphatase and 1 mg/ml Fast Red-TR salt. Sections were counterstained with Harris haematoxylin (BDH) and mounted in Glycergel (Dako Ltd, High Wycombe, UK). System and specificity controls were carried out on all staining runs.

Counting methods

Slides were read by two observers in a blind fashion, in coded random order using an Olympus BH2 microscope (Olympus Optical Company Ltd, Tokyo, Japan). The submucosal area for counts was quantified by use of a computer-assisted graphics tablet visualized by a side-arm attachment to the microscope. Counts were expressed per mm^2 of cross-sectional subepithelial surface. The interobserver variability was < 10%.

Data analysis

Non-parametric analysis of variance (Kruskal–Wallis method) was used to determine significant variance among the three groups. When a significant variance was found, a Mann–Whitney *U*-test was used to compare individual groups. Regression analysis was performed by Spearman's rank correlation. A *P*-value of < 0.05 was accepted as significant. Data were analysed with the MINITAB standard statistical package (Minitab Inc., State College, PA).

RESULTS

Bronchial responsiveness to ACh

ACh caused a significantly greater increase in R_L at 10^{-3} ($P < 0.01$) and 10^{-2} ($P < 0.05$) M/l concentrations in the group that received i.p. sensitization and aerosol exposure of OVA, compared with saline-exposed animals (Fig. 1a). There was also a shift in the dose–response curve of sensitized and exposed animals as expressed by a significant decrease in their PC_{50} and PC_{100} values compared with saline-exposed controls ($P < 0.05$) (Fig. 1b). There was no significant difference in the mean baseline R_L between OVA- and saline-exposed rats.

BAL cell profile

In the sensitized/OVA-exposed group there was a significant increase in eosinophils ($P < 0.03$), neutrophils ($P < 0.01$) and lymphocytes ($P < 0.02$) compared to saline-exposed control rats. There was no significant difference in the number of alveolar macrophages, eosinophils, lymphocytes and neutrophils recovered from naive and i.p. sensitized/saline-exposed animals (Fig. 2).

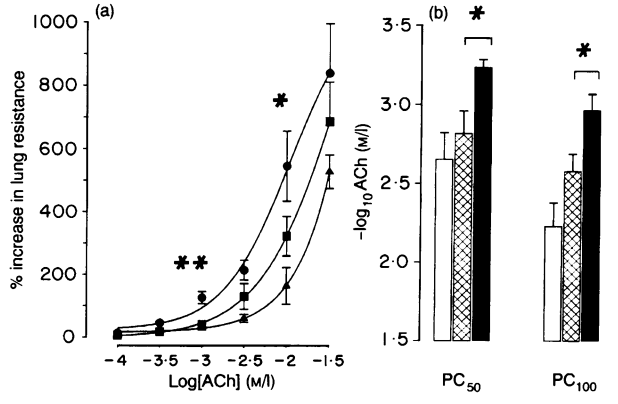

Figure 1. (a) Effect of i.p. sensitization and inhalational exposure to OVA on airway responsiveness to ACh. Three groups were studied: non-sensitized/non-exposed rats (▲; $n = 8$); OVA-sensitized/saline-exposed rats (■; $n = 7$); OVA-sensitized/OVA-exposed rats (●; $n = 9$). $*P < 0.05$; $**P < 0.01$ between saline- versus OVA-exposed rats. Results expressed as mean ± SEM. (b) Concentrations of ACh needed to increase lung resistance by 50% and 100% above baseline. (□) Non-sensitized/non-exposed rats; (▨) OVA-sensitized/saline-exposed rats; (■) OVA-sensitized/OVA-exposed rats. $*P < 0.05$.

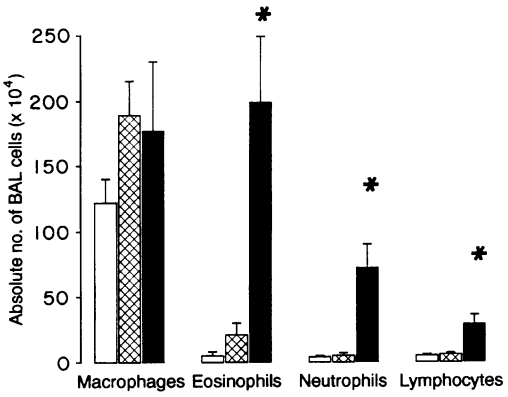

Figure 2. Effect of i.p. sensitization and inhalational exposure to OVA on cellular content of BAL in Brown–Norway rats. Absolute numbers of cells were calculated by using the differential counts on cytospin preparations stained with May–Grünwald–Giemsa, and the total numbers of cells recovered from the lavage fluid. (□) Non-sensitized/non-exposed rats ($n = 8$); (▨) OVA-sensitized/saline-exposed rats ($n = 7$); (■) OVA-sensitized/OVA-exposed rats ($n = 9$). $*P < 0.05$ (OVA-sensitized/saline-exposed versus OVA-sensitized/OVA exposed). Results expressed as mean ± SEM.

Lymphocyte subsets in the airways

We used sections of bronchus-associated lymphoid tissue (BALT) in order to validate our immunohistological results. There was no positive staining in the IgG-labelled control sections (Fig. 3a). B cells ($CD45RC^+$) and T cells ($CD2^+$) were located in mutually exclusive zones (Fig. 3b, c). Anti-CD2, anti-CD4 and anti-CD8 antibody labelling gave a specific membrane staining of lymphocytes in BALT (Fig. 3c, d, e).

The rat lymphocyte surface in the airway mucosa also gave specific staining with anti-CD2, anti-CD4 and anti-CD8 antibodies (Fig. 3f, g, h). The numbers of $CD8^+$ cells were significantly elevated in the group sensitized and exposed to OVA compared to saline-exposed rats (42·1 ± 14·0 versus 114·3 ± 21·6; $P < 0.05$). Rats sensitized and exposed to OVA showed significantly increased numbers of $CD25^+$ cells in the mucosal and submucosal tissue compared with the saline-exposed control naive animals (Figs 3h and 4). There were no positive cells labelled with anti-CD25 antibodies in the BALT tissue. For a positive control for CD25, we used cytospins prepared from concanavalin A (Con A)-stimulated peripheral blood lymphocytes.

Eosinophil counts in the mucosal tissue

Animals that were sensitized and exposed to OVA had significantly higher numbers of eosinophils in the airway submucosa than naive controls (805·0 ± 236·5 and 82·1 ± 27·1, respectively; $P < 0.003$) (Fig. 4). The increase in eosinophil numbers in sensitized rats following exposure to

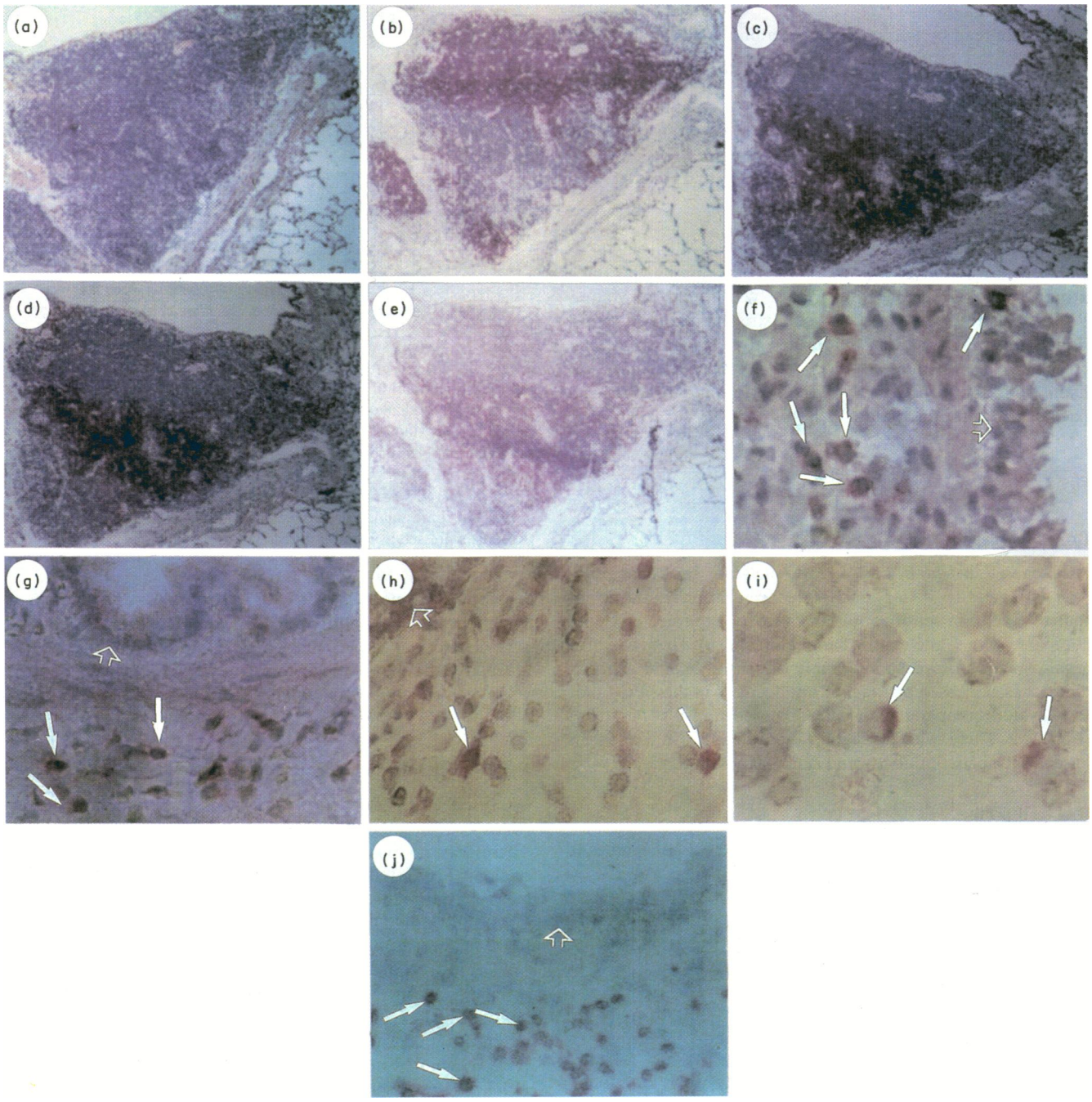

Figure 3. Labelling of lung tissues with (mAb). (a, b, c, d and e) Typical examples of serial sections of bronchus-associated lymphoid tissue showing localization of anti-CD45RC-labelled B cells (b) and anti-CD2-, anti-CD4- and anti-CD8-labelled T cells (c, d and e, respectively). A mixture of IgG1a/b and IgG2 was used as negative control (a). Positive staining for CD2 (f), CD4 (g), CD8 (h), CD25 (i) and BMK-13 (j) in submucosal tissue of airways are also shown as indicated by the arrows. Closed arrows indicate examples of positive staining in individual cells, while the open arrows indicate the position of the airway epithelium. Magnification factors: (a–e) × 100; (f–h) and (j) × 400; (i) × 1000.

OVA was not significant compared with sensitized and saline-exposed animals.

Relationships between R_L, eosinophils and T-cell subsets

There was a significant positive correlation between the percentage increase in R_L at an ACh concentration of 10^{-3} M/l and the numbers of $CD4^+$ ($r = 0{\cdot}77$; $P < 0{\cdot}05$), $CD8^+$ ($r = 0{\cdot}71$; $P < 0{\cdot}05$), $CD25^+$ ($r = 0{\cdot}92$; $P < 0{\cdot}001$) and $BMK13^+$ ($r = 0{\cdot}72$; $P < 0{\cdot}03$) cells in the animals sensitized and exposed to OVA, but not in the saline-exposed or naive controls. The number of MBP^+ cells also

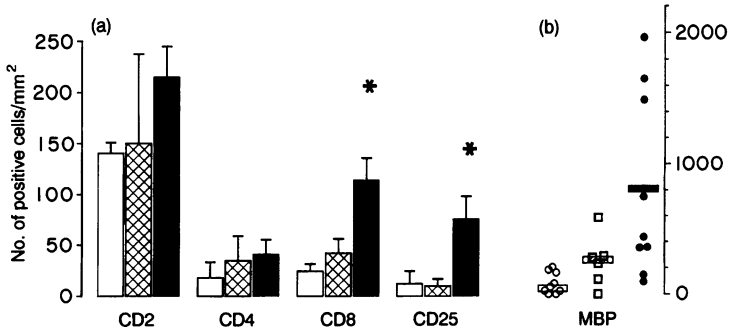

Figure 4. (a) Effects of sensitization and exposure to ovalbumin on lymphocyte subpopulations in the airway mucosal tissue. (□) Non-sensitized/non-exposed rats (n = 8); (▨) OVA-sensitized/saline-exposed rats (n = 7); (■) OVA-sensitized/OVA-exposed rats (n = 9). *P < 0·05. (OVA-sensitized/saline-exposed versus OVA-sensitized/OVA-exposed). (b) Effects of sensitization and exposure to OVA on eosinophil cell influx into the airway mucosal tissue as assessed by staining using BMK-13. (○) Non-sensitized/non-exposed rats (n = 8); (□) OVA-sensitized/saline-exposed rats (n = 7); (●) OVA-sensitized/OVA-exposed rats (n = 9). Horizontal bars indicate mean values.

correlated with CD25 expression (r = 0·71; P < 0·05) in this group.

DISCUSSION

We have shown that, following a single allergen exposure with OVA, there is a significant accumulation of activated T cells expressing CD25 marker, and of $CD8^+$ T cells, together with an increase in the number of eosinophils in the airway submucosa of the allergic rat. No significant increases in $CD4^+$ T cells were observed. Sensitization alone did not result in an increase in total or activated T cells and eosinophil counts. Moreover, we have demonstrated a significant correlation between the degree of airway response to ACh, eosinophilic influx, as measured by MBP^+ cells, and the number of activated T cells ($CD25^+$). We have not included another control group in this study, that of non-sensitized rats exposed to OVA aerosol, because in a previous detailed study to characterize this rat model there was no evidence of bronchial hyperresponsiveness and airway inflammation, as assessed by BAL.[6]

Although it is difficult to extrapolate results from other animals to humans, the Brown–Norway rat model appears to have several characteristics in common with human allergic asthma. In this rat model, high levels of IgE in response to OVA have been reported,[11,12] and exposure to OVA results in a high proportion of early and late responses[11] and in airway hyperresponsiveness.[6] In addition, there is a concomitant influx of eosinophils and lymphocytes in BAL fluid, together with an increase in activated lymphocytes bearing $CD25^+$.[7] In our present study, we demonstrated an influx of eosinophils and lymphocytes in the airway tissues, in particular $CD8^+$ and $CD25^+$ T cells in this rat model. Comparative data in human allergic asthma following allergen challenge is scanty, but data obtained from limited bronchial mucosal biopsy samples from stable human asthma patients have demonstrated that activated ($CD25^+$) T cells have been observed in bronchial biopsies of asthmatic patients, and these correlated with both the numbers of activated eosinophils and disease severity.[3] Following allergen challenge, Gonzalez *et al.*[13] reported relative increases in $OKT8^+$ ($CD8^+$ cells) in BAL fluid of patients who exhibited a single early bronchoconstrictor response, compared with dual early- and late-phase responders. Other studies have reported a selective increase in $CD4^+$ T cells in BAL fluid.[14,15] In one study, allergen challenge of mild allergic asthmatics did not result in significant changes in $CD3^+$, $CD4^+$ and $CD8^+$ lymphocyte populations; there was, however, significant increases in $CD25^+$ T cells.[16] Our data clearly establish that sensitization followed by saline exposure is not sufficient to increase the numbers of CD2, CD4, CD8 and CD25 T cells in airway mucosal tissue. However, in a previous study, we found that sensitization resulted in a significant increase in CD25 expression, found mostly on $CD4^+$ T cells recovered by BAL.[17]

As in human asthma, there are few studies in animal models of the disease that have examined the lymphocytic cellular infiltration in the airway wall after antigen challenge. In the guinea-pig, sensitization by exposure to OVA aerosol increased mucosal T-cell numbers, which consisted entirely of $CD8^+$ T cells.[18] Similar findings have been reported in murine spleen and in the rat respiratory lymph nodes following sensitization.[19,20] Following re-exposure to allergen, a rise in $CD3^+$ T cells was observed in the sensitized guinea-pig airway, which was $CD8^-$; an increase in submucosal eosinophils was also observed.[18] However, these results are at variance with those reported by Lapa e Silva *et al.*,[21] who showed an increase in $CD4^+$ T cells in guinea-pig airway mucosa on sensitization, with no further increase following challenge. Using a technique for retrieving inflammatory cells by tissue mincing and digestion with collagenase, Renzi *et al.*[22] reported that allergen challenge of Brown–Norway rats was associated with a predominantly neutrophilic cellular influx into the large and small airways, as well as parenchyma 8 hr after allergen challenge. Animals with a late response had a significantly lower cellular yield from the large and small airways and fewer lymphocytes and eosinophils. There was a difference in lymphocyte subsets, with an increase in the percentage of suppressor cells ($CD8^+$), and a lower helper ($CD4^+$) to suppressor ($CD8^+$) ratio.[22] These observations are in agreement with those we have described here, especially the significant increase in $CD8^+$ T-cells. However, rather surprisingly, no increase in tissue eosinophils was found in the study of Renzi *et al.*[22]

We found a significant increase in the number of activated T cells bearing the CD25 marker. Although the precise T-cell subtype ($CD4^+$ or $CD8^+$) bearing the CD25 was not investigated in our study, the fact that only $CD8^+$ T cells increased after allergen challenge does not exclude the possibility that $CD8^+$ T cells may also bear the CD25 marker.

The mechanisms by which the number of $CD8^+$ T cells increases in rat airways following allergen challenge are unclear. In addition, their role in the pathogenesis of airway hyper-responsiveness remains unclear, although their role in IgE production has recently been addressed. In the rat, specific protection against allergic sensitization by repeated exposure to allergen has been shown to be mediated by $CD8^+$ suppressor/cytotoxic T cells.[20,23] It is possible that $CD8^+$-derived interferon-γ (IFN-γ) could directly suppress class switching to IgE in B cells.[24] Another explanation could be the regulation of $CD4^+$ T-cell development by IFN-γ by suppressing the development of T-helper type-2 (Th2) cells, thus favouring a Th1 cell pattern.[25] Transfer studies of CD8 T cells in the allergic mouse model have shown that these cells may not only suppress IgE responses but can also inhibit airways hyper-responsiveness.[19] Thus, it is possible that $CD8^+$ T cells in the airways of Brown–Norway rats following allergen exposure may represent mechanisms by which down-regulation of induced airways hyper-responsiveness is affected. However, the possibility that there are functional subsets of $CD8^+$ T cells,[26] similar to those described for $CD4^+$ T cells, raises the likelihood of different profiles of cytokines being expressed, which may ultimately determine the final allergic inflammatory response and associated airway hyper-responsiveness.

We used a mAb to human MBP (BMK-13), which has been shown to cross-react with rat MBP,[10] in order to quantify the number of infiltrating eosinophils. The only other cell which may bind BMK-13 is the basophil, because it contains small amounts of MBP. However, we have not documented the presence of any basophils in this inflammatory response in the lungs. Furthermore, the intensity of BMK-13 staining indicates a strong positive immunoreactivity that can only be seen in the eosinophils. A strong correlation between the airway response to ACh and the numbers of $CD4^+$, $CD8^+$, $CD25^+$ and MBP^+ cells was found. In addition, there was a strong correlation between the number of MBP^+ cells and the number of T cells expressing CD25. These data support the hypothesis that activated T cells may be responsible for the eosinophil influx, which in turn may underlie the increased responses to ACh. MBP has the capacity to induce airway hyper-responsiveness when instilled directly into the airways.[27,28] Although it has been suggested that airway epithelial damage may be caused by eosinophils and may contribute to airway hyper-responsiveness,[29] we found no evidence for epithelial damage by light microscopy.

In summary, we have demonstrated, in the sensitized Brown-Norway rat, that a single allergen exposure leads to an increase in airway hyper-responsiveness to ACh associated with an increase in airway submucosal eosinophils, $CD8^+$ and $CD25^+$ T cells, but not $CD4^+$ and $CD2^+$ T cells. These results, which bear certain similarities to those reported from studies in asthmatic patients, support the premise that the Brown–Norway rat is a most pertinent model for human asthma, which lends itself to further examination of the role of lymphocytes and eosinophils in airway hyper-responsiveness. The potential role for $CD8^+$ T cells in the pathogenesis of airway hyper-responsiveness induced by single allergen challenge requires further investigation.

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